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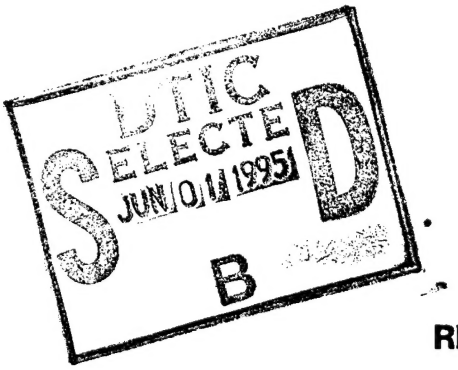
INHIBITION OF RABBIT SPERM CELL HYPERACTIVATED MOTILITY BY METALLIC IONS IN TOXICOLOGICAL TESTING



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13. ABSTRACT *(Maximum 200 words)*

Rabbit sperm cells developed hyperactivated motility when incubated in medium M. Hyperactivated but not normal progressive motility was inhibited by lead and cadmium, two metals implicated in fertility dysfunction. A metal of military interest, Cr^{+6}, had a similar outcome on rabbit sperm, suggesting that it may also have the same property as cadmium and lead. Two metals, zinc and mercury, without fertility effects did not suppress hyperactivated motility. The rabbit sperm-medium M-hyperactivated motility system may have a role in the *in vitro* assessment of the antifertility effects of chemicals.

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PREFACE

The work described in this report was authorized under Project No. F8J2-10-005. This work was started in November 1990 and completed in August 1992.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," National Institute of Health Publication No. 86-23, 1985, as promulgated by the committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council (Washington, DC). These investigations were also performed in accordance with the requirements of AR 70-18, Laboratory Animals, Procurement, Transportation, Use, Care, and Public Affairs.

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QUALITY ASSURANCE

This study, governed by Protocol Number 210910430000, was examined for compliance with Good Laboratory Practices as published by the U.S. Environmental Protection Agency in 40 CFR Part 792 (effective 18 September 1989). The dates of all inspections and the dates the results of those inspections were reported to the Study Director and management were as follows:

Phase Inspected	Date Inspected	Date Reported to Study Director/Management
Videotaping	05 Nov 1991	05 Nov 1991
Final Report	18 July 1994	15 Sept 1994

To the best of my knowledge, the methods described in this report were the methods followed during the study as indicated by the raw data found in the laboratory notebook. The report was determined to be an accurate reflection of the raw data recorded.

Kenneth P. Cameron
Quality Specialist
Life Sciences Department

15 Sept 1994
Date

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TABLES

INHIBITION OF RABBIT SPERM CELL HYPERACTIVATED MOTILITY BY METALLIC IONS IN TOXICOLOGICAL TESTING

1. INTRODUCTION

The rabbit sperm cell is a suitable system for the *in vitro* assay of chemical cytotoxicity.[1] Work in progress points to this sperm's potential use for *in vitro* testing of the fertility effects of chemicals. A recent study shows that T6 is the medium of choice[2] for rabbit sperm cell culture; but, this medium, in common with DM and other media frequently used to culture sperm cells, contains carbonate, phosphate, sulfate, and organic acids, and requires the use of an atmosphere containing 5% CO_2. These conditions are not suitable for testing volatile compounds and compounds that form insoluble carbonates, phosphates, sulfates, or salts of low solubility with organic acids. In addition, rabbit sperm develop hyperactivated motility only after lengthy incubation in these media, an impediment to the application of the system for an *in vitro* fertility assay based on the inhibition of hyperactivated motion. A new simple salts medium (medium M) in which rabbit sperm cells develop hyperactivated motility in 0.5-1 hr and which supports cellular motion for 20 hr has been developed.[3] This medium contains only chlorides, glucose, and bovine serum albumin (BSA) and is buffered by TRIS-HCl. To determine the suitability of the medium for *in vitro* testing, the effects of $PbCl_2$, $CdCl_2$, $HgCl_2$, $ZnCl_2$, and $K_2Cr_2O_7$ on the motion of rabbit sperm cells were studied. The metals Pb, Cd, Zn, Hg, and Cr were chosen for study because their effects on male reproduction are either known or are under study, and because their cations form insoluble carbonates, phosphates, or sulfates. The experiments conducted are described herein.

2. MATERIALS AND METHODS

2.1 Animals.

New Zealand white rabbits were individually housed in standard rabbit cages in a room maintained at 75 °F and 50 ± 10% relative humidity (RH) with a 12-hr light/dark cycle. Standard certified laboratory rabbit chow and water were available *ad libitum*.

2.2 Collection and Purification of Sperm Cells.

Semen was collected, and sperm cells were purified by centrifugation through a discontinuous Percoll gradient previously described.[4] Seminal plasma was also removed by

diluting semen with 2 mL of warm medium and centrifugating it at room temperature for 5 min at 350 g. The pellet was washed once and resuspended in warm medium.

2.3 Incubation of Sperm Cells.

Purified cells were resuspended in medium at a concentration of 5-10 x 10^6/mL and incubated at 37 °C in air when medium M was used or 5% CO_2 in air when medium T6 was used. Cells were exposed to three concentrations of the test compounds together with a control without a test compound. The compounds and concentrations were: $ZnCl_2$, 0.0002M, 0.0001M, 0.00005M; $K_2Cr_2O_7$, 0.000005M, 0.0000025M, 0.000001M; $CdCl_2$, 0.0001M, 0.00005M, 0.00002M; $HgCl_2$, 0.000001M, 0.0000005M, 0.0000002M, and $PbCl_2$, 0.000025M, 0.00001M, 0.000005M. Each experiment was carried out several times with cells from different rabbits to assess variation in response among rabbits. Bovine serum albumin formed a flocculent precipitate with $ZnCl_2$ when used at 3 mg/mL but not at 2 mg/mL. Experiments with this compound were carried out at the lower BSA concentration. At 0, 0.5, 1, 2, and 4 hr after adding the test compounds, a 5-7-μL drop of the cell suspension was placed either into a warm 20-μm deep chamber or a 10-μL drop was put into a warm 20-μL MicroCell slide (Fertility Technologies, Natick, MA). Observation of cellular motility was carried out at 100x under negative phase contrast with an Olympus microscope equipped with a warming stage. Some experiments were videotaped[1] for later analysis.

3. RESULTS

Changes in Motility Characteristics.

Rabbit sperm cells suspended in T6 medium moved either progressively or in circles over a 6-hr period; but, few cells developed hyperactivated motility. Adding $CdCl_2$ at 0.0001M did not alter the motility characteristics. Motility was inhibited in 2 hr when the salt was added at a concentration of 0.001M and in 4 hr at a concentration of 0.0005M; but, cells remained motile over 6 hr at a concentration of 0.0002M. A completely different result was obtained when incubation was carried out in medium M. Motility was inhibited in 1 hr by $CdCl_2$ at a concentration of 0.0002M, demonstrating that in T6 medium, Cd^{2+} is sequestered from the solution even though a precipitate was not readily visible. Hyperactivated motility also developed in medium M, and the extent of hyperactivated motility decreased as a function of time of exposure and concentration of $CdCl_2$ (Table 1). The response of cells to $CdCl_2$ varied according to the cell source. The low concentrations of $CdCl_2$ that inhibited hyperactivated motility had no apparent effect on the motility of non-hyperactivated cells as these cells remained motile after hyperactivated motion ceased.

Table 1. Effect of $CdCl_2$ on Rabbit Sperm Cell Motility.

Rabbit	Conc.	Incubation Time (Hour)				
		0	0.5	1	2	4
038	0	≥50*,4#	≥60,5	≤45,5	≤35,5	≤20,4
	H		≤50,4	≤20,3	0	0
	M		≥60,4	≤30,4	≤25,3	0
	L		≥60,4	≤40,3	≤25,3	≤20,3
213	0	0	≥15,3	≥30,3	≤ 5,3	≤ 2,3
	H		≤10,3	≤ 1,3	0	0
	M		≥10,3	≤ 5,3	0	0
	L		≥30,3	≥ 5,3	0	0
967	0	≥30,3	≥50,3	≤35,4	≥30,4	≤ 5,3
	H		≤30,3	≤30,3	0	0
	M		≥50,3	≤35,4	≤ 5,3	0
	L		≥50,4	≥35,4	≤30,3	≤ 5,3
793	0	0	≤ 5,3	≥40,4	≤25,4	≤ 5,3
	H		≤ 3,3	≤15,3	≤ 3,3	≤ 2,3
	M		≤ 5,3	≤30,3	≥ 5,3	≤ 2,3
	L		≥ 3,3	≤40,3	≤20,3	≥ 2,3
772	0	0	≤ 2,3	≥10,4	≥10,3	≤ 3,3
	H		"	≤ 5,3	0	0
	M		"	≤10,3	≤ 3,3	0
	L		"	≤ 6,3	≥ 5,3	≤ 3,3
967	0	≥50,3	≥50,3	≤50,3	≤ 2,3	≤ 2,3
	H1		N.M.			

A suspension of sperm cells in medium M was incubated at 37 °C with $CdCl_2$. At the indicated time intervals, a drop of the cell suspension was placed into a warm 20 μm deep chamber, and cell motion was observed under negative phase contrast with an Olympus microscope equipped with a heated stage.

* Percent of motile sperm cells that are hyperactivated based on microscopic observation at 400x under negative phase contrast.

Quality of motion of total sperm cell population ranked from 1 (low) to 5 (high).

N.M. Non motile

H = 0.1mM, M = 0.05mM, L = 0.002mM; H1 = 0.2mM

Lead chloride did not form a visible precipitate with T6 medium when the chloride was added at a concentration <0.0005M, and cell motility was not affected in this medium. However, hyperactivated motility was inhibited when sperm cells were incubated in medium M with $PbCl_2$ (Table 2). Here also, motility of non-hyperactivated sperm cells did not appear to be changed by concentrations of $PbCl_2$ that inhibited hyperactivated motion. Similar results were obtained when sperm cells in medium M were exposed to $K_2Cr_2O_7$ (Table 3). As shown in Tables 2 and 3, response to the two compounds differed among rabbits.

The response of sperm cells to $HgCl_2$ was quite varied. Hyperactivated motility of cells from some rabbits was not inhibited by this compound at a concentration of 0.000001M (Table 4, rabbit 967); whereas, the same concentration completely inhibited motility in others (Table 4, rabbit 213). At a concentration in which cells remained motile, $HgCl_2$ did not seem to inhibit hyperactivated motility (Table 4, rabbits 213 and 712).

The action of $ZnCl_2$ was also variable. Motility, hyperactivated or non-hyperactivated, of cells from some rabbits was unaffected. Hyperactivated motility of cells from other rabbits was not reduced after short incubation; but, longer incubation inhibited both types of motion (Table 5). This result may be due, in part, either to the lower concentration of BSA used in experiments with $ZnCl_2$ or to BSA denaturation by $ZnCl_2$. A precipitate was not visible in the sperm cell suspension; but, denatured BSA, although not visible, may have affected sperm viability.

4. DISCUSSION AND CONCLUSION

Results show that medium M is suitable for use in assessing the effects of metal cations on sperm cell motion. The concentrations of all the metal cations used in the study encompass the ranges found in the body fluids of human males exposed to the metals.[5-8] Lead is suspect as the cause of fertility problems in exposed workers.[5,9,10] At high exposure levels, spermatogenesis is disturbed. Cadmium is a potent testicular poison, and exposure to this metal impairs spermatogenesis. However, this does not adequately explain fertility consequences following exposure to the metals.[9,10] Inhibition of hyperactivated motility demonstrated with the two metals in the present study provides one explanation for fertility effects associated with the metals when motility of seminal sperm cells falls within the normal range. The ability of Cr^{+6} to inhibit hyperactivated motility suggests that exposure to this metal could result in disturbance of the reproductive function. Epidemiological studies suggest there is cause for

Table 2. Effect of $PbCl_2$ on Hyperactivated Motility

Rabbit	Conc.	Incubation Time (Hour)				
		0	0.5	1	2	4
772	0	≤ 5*,3#	≤10,3	≤25,3	≤ 1,3	0
	H		≤ 1,3	≤ 1,3	≤ 1,3	0
	M		≤ 1,3	≤ 1,3	≤ 1,3	0
	L		≤ 5,3	≤ 3,3	≤ 1,3	0
712	0	≤ 2,3	≤15,3	≥40,3	≤40,3	≤ 5,3
	H		≤ 5,3	≤40,3	≤ 5,3	≤ 5,3
	M		≤15,3	≤40,3	≤30,3	≤ 5,3
	L		≤15,3	≤40,3	≤40,3	≤ 5,3
864	0	≤ 2,2	≤15,4	≤10,4	≤ 2,3	0
	H		≤ 1,3	0	0	0
	M		≤ 5,3	≤ 5,3	0	0
	L		≤ 5,4	≥ 5,4	0	0
779	0	≤ 2,2	≤ 2,3	≤10,3	≤ 5,3	0
	H		0	0	0	0
	M		-	≤ 1,3	0	0
	L		-	≤ 3,3	0	0
038	0		≤ 5,3	≥50,5	≥35,4	≤30,4
	H		≤ 5,2	≤50,4	≤30,3	≤20,3
	M		≤ 5,3	≤50,3	≤30,3	≥20,3
	L		≤ 5,3	≤50,4	≥30,3	≥20,3

A suspension of sperm cells in medium M was incubated at 37 °C with $PbCl_2$. At the indicated time intervals, a drop of the cell suspension was placed into a warm 20 μm deep chamber, and cell motion was observed under negative phase contrast with an Olympus microscope equipped with a heated stage.

* Percent of motile sperm cells that are hyperactivated based on microscopic observation at 100x under negative phase contrast.

Quality of motion of total sperm cell population ranked from 1 (low) to 5 (high).

H = 0.025mM, M = 0.01mM, L = 0.005mM

Table 3. Effect of $K_2Cr_2O_7$ on Hyperactivated Motility.

Rabbit	Conc.	Incubation Time (Hour)				
		0	0.5	1	2	4
038	0	≤ 3*,2#	≥50,3	≥40,3	≥30,4	≥30,4
	H	-	≤10,3	≤ 5,3	≤ 5,3	≤ 3,3
	M	-	≤30,3	≤10,3	≤25,3	≤20,3
	L	-	≤40,3	≤40,3	≤30,3	≤30,4
793	0	0	≤ 5,3	≥15,3	≤15,3	0
	H	-	0	≤10,3	≤ 1,3	0
	M	-	≤ 5,4	≤10,3	≤ 2,3	0
	L	-	≤ 5,3	≥20,3	≤ 5,3	0
712	0	≤ 3,2	≥20,4	≥20,4	≥20,4	≥20,4
	H	-	0	≤ 5,3	≤ 5,3	0
	M	-	-	≤12,3	≤10,3	0
	L	-	-	≤15,3	≤15,4	≤10,3
967	0	≤40,3	≥50,4	≥20,3	≥20,3	≥10,3
	H	-	≤50,3	≤30,3	≤10,3	≤ 2,3
	M	-	≤50,3	≤30,3	≥10,3	≤ 2,3
	L	-	≤50,3	≥35,3	≥10,3	≤ 5,3
772	0	≥20,4	≥25,4	≥25,4	≥15,4	≥ 5,4
	H	-	≤ 5,4	≤ 5,3	≤ 5,3	0
	M	-	≤20,3	≤20,3	≥10,3	0
	L	-	≤20,3	≤20,3	≥10,3	≤ 2,3

A suspension of sperm cells in medium M was incubated at 37 °C with $K_2Cr_2O_7$. At the indicated time intervals, a drop of the cell suspension was placed into a warm 20 μm deep chamber, and cell motion was observed under negative phase contrast with an Olympus microscope equipped with a heated stage.

* Percent of motile sperm cells that are hyperactivated based on microscopic observation at 100x under negative phase contrast.

Quality of motion of total sperm cell population ranked from 1 (low) to 5 (high).

H = 0.005mM, M = 0.0025mM, L = 0.001mM

Table 4. Effect of $HgCl_2$ on Hyperactivated Motility

Rabbit	Conc.	Incubation Time (Hour)				
		0	0.5	1	2	4
864	0	0	≤ 5,3	≥30,3	≤ 5,3	0
	H	-	≤ 1,1	0	0	0
	M	-	≤ 1,3	≤10,3	≤ 3,3	0
	L	-	≥ 5,3	≤ 5,3	≤ 3,3	0
038	0	0	≥60,5	≥60,5	≥35,4	≥30,4
	H	-	≤ 3,2	0	0	0
	M	-	≥30,3	≥10,3	≥10,3	≥10,3
	L	-	≥50,4	≤60,4	≤30,3	≥25,3
213	0	≤ 3*,3#	≥20,4	≥25,3	≥15,3	≥10,3
	H	-	0	0	0	0
	M	-	≤15,4	≤15,3	≥15,3	≤ 5,3
	L	-	≥20,4	≤25,3	≥15,3	≤10,3
712	0	-	≥15,2	≥25,3	≤10,3	0
	H	-	≤10,2	≥ 5,3	≤ 5,3	0
	M	-	≥20,2	≥20,3	≥ 5,3	0
	L	-	≥25,2	≤20,3	≤ 5,3	0
772	0	-	≥25,4	≥10,3	≤10,3	0
	H	-	≥15,3	≤ 3,3	≤ 2,3	0
	M	-	≥20,4	≤ 3,3	≤ 3,3	0
	L	-	≥25,4	≥ 3,3	≥ 3,3	0
967	0	≤ 2,3	≥60,4	≥50,3	≤30,3	0
	H	-	≥60,4	≤50,3	≤20,3	0
	M	-	≥60,4	≤50,3	≤20,3	0
	L	-	≥60,4	≤50,3	≤20,3	0
793	0	-	-	≥30,4	≥20,3	≤ 5,3
	H	-	-	≥30,4	≥20,3	≤ 5,3
	M	-	-	≥30,4	≥20,3	≤ 5,3
	L	-	-	≥30,4	≥20,3	≤ 5,3

A suspension of sperm cells in medium M was incubated at 37 °C with $HgCl_2$. At the indicated time intervals, a drop of the cell suspension was placed into a warm 20 μm deep chamber, and cell motion was observed under negative phase contrast with an Olympus microscope equipped with a heated stage.

* Percent of motile sperm cells that are hyperactivated based on microscopic observation at 100x under negative phase contrast.

Quality of motion of total sperm cell population ranked from 1 (low) to 5 (high).

H = 0.001mM, M = 0.0005mM, L = 0.0002mM

Table 5. Effect of $ZnCl_2$ on Hyperactivated Motility

Rabbit	Conc.	Incubation Time (Hour)				
		0	0.5	1	2	4
038	0	≤ 2*,2#	≥50,3	≥50,3	≤20,3	≤20,3
	H	-	≥50,3	≥50,3	≤20,3	≤20,3
	M	-	≥50,3	≥50,3	≤20,3	≤20,3
	L	-	≥50,3	≥50,3	≤20,3	≤20,3
972	0	≤ 5,3	-	≥15,4	≤ 5,3	≤ 5,3
	H		-	≤10,4	≤ 5,3	≤ 5,3
	M	-	-	≤10,4	≤ 5,3	≤ 5,3
	L	-	-	≤10,4	≤ 5,3	≤ 5,3
712	0	0	≤20,3	≥10,3	≤ 5,3	0
	H		-	≤ 5,3	≤2,3	0
	M	-	≤20,3	≥10,3	≤ 2,3	0
	L	-	≤20,3	≥10,3	≤ 2,3	0
793	0	0	≤ 5,3	≤25,3	≤20,3	0
	H	-	≤ 5,3	≤10,3	≤10,3	0
	M	-	≤10,3	≤10,3	≤10,3	0
	L	-	≤20,3	≤15,3	≤10,3	0
772	0	0	≥20,2	≥20,3	≥10,3	0
	H	-	≥20,3	≥20,3	≤ 2,1	0
	M	-	≤20,3	≥20,3	≤ 3,3	0
	L	-	≤20,3	≥20,3	≤ 5,3	0

A suspension of sperm cells in medium M was incubated at 37 °C with $ZnCl_2$. At the indicated time intervals, a drop of the cell suspension was placed into a warm 20 μm deep chamber, and cell motion was observed under negative phase contrast with an Olympus microscope equipped with a heated stage.

* Percent of motile sperm cells that are hyperactivated based on microscopic observation at 100x under negative phase contrast.

Quality of motion of total sperm cell population ranked from 1 (low) to 5 (high).

H = 0.2mM, M = 0.1mM, L = 0.05mM

concern.[11] Zinc is present in high concentration in seminal plasma of mammals and has little effect on fertility.[12] It is significant that exposure to the metal generally had little consequence for hyperactivated motility of rabbit sperm cells. Hyperactivated motility was not suppressed by Hg^{2+} either in nonsensitive rabbit sperm cells or at concentrations that did not inhibit motility in sensitive cells, suggesting that at either low levels or with nonsensitive cells, inorganic mercury is relatively benign with respect to fertility impairment. The estimation of the decrease in hyperactivated motility was based on subjective visual observation by the same worker in all experiments, and the trend in hyperactivation suppression was found in the 6-7 replicates carried out with each compound studied. An objective measure of hyperactivated motility, under development, will provide a firmer basis for the above conclusions.

The inhibition of hyperactivated motility by the two metals implicated in the impairment of fertility, the overall lack of effect of another regarded as without impact on fertilization, together with the prediction of the consequence for fertilization by exposure to a fifth, demonstrate the utility of the rabbit sperm cell-medium M-hyperactivated motility system for the in vitro assessment of the fertility effects of chemicals. By adjusting the concentration range of the test compound, an assessment of the cytotoxicity of the chemical may also be obtained in the same experiment using methods previously described.[1]

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